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Cultured Neuron Probe

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General Introduction

Our aim is to develop a cultured neuron probe. This consists of a silicon structure into which individual dissociated neurons can be placed, and which can be inserted into an intact nervous system. Furthermore, within the structure each neuron is in close proximity to an electrode, by means of which it can be stimulated, or its activity can be recorded, through electrical leads which connect to external electronics. It is hoped that neurons in the probe will synaptically integrate with the host nervous system, to provide a highly specific, relatively non-invasive, two-way communication channel. If this occurs, the methodology has important possibilities for neural prostheses. The goal of this project is to perform initial experiments to establish the feasibility of communication by means of a neuron probe. The tissue we have chosen in which to initially implant the probe is the rat hippocampus. If initial studies are successful, probes will be designed and implanted for communication with sensorimotor cortex.

The neuron probe we plan to fabricate will be made of micromachined silicon and will have sixteen electrodes: one conventional electrode to detect activity during placement of the probe, and fifteen within wells into which neurons will be placed. Its configuration will be similar to that of passive multielectrode probes which have been developed. It will be implanted when the cultured neurons are very young, and after a time of weeks it is hoped that they will have survived and made two-way synaptic connections. By stimulation and recording in the host and probe, we will test for the existence of such connections. An essential feature of the experiments is that the viability of the implanted neurons will be independently determined over time by stimulating them and recording their resulting action potentials.

Introduction

This report covers the first quarter of a new contract, following three years of work previously funded by the Neural Prosthesis Program. At the end of that period, the following progress had been made, setting the stage for the ongoing work under this contract.

1. Dummy probes had been fabricated (with wells but without electrodes), and techniques developed for successfully inserting neurons into wells.

2. Trial probe insertions were made into rat hippocampus. Histological techniques for sectioning and staining tissue in order to determine the extent of cell outgrowth were developed. One possible outgrowing process was observed.

3. Dummy neurochips (without electrodes) were fabricated, with wells in a 4 x 4 square array, for *in vitro* tests.

4. Cell outgrowth by both sympathetic neurons and hippocampal pyramidal cells was observed from wells in dummy neurochips.

5. Complete neurochips with electrodes were fabricated and the electrodes platinized and found to have satisfactory low impedances.

6. Electronics and computer software for selective stimulation and recording from sixteen neurons simultaneously had been completed.

7. Sudies of staining of dissociated neurons were begun, to determine whether cells and their outgrowing processes could be labelled for periods of weeks or months after probe implantation.

As this new contract begins, we are regaining momentum after a slowdown during three months between the two contracts. At Caltech, we have lost our technician, Tim Denison, and gained a biology graduate student, Hannah Dvorak. We have hired two postdoctoral fellows to each spend half

time on this project, Mike Maher, a condensed-matter experimental physicist from Cornell, and Steve Potter, a neurobiologist from Irvine. They will arrive during the next quarter. At Rutgers, a Research Associate from the former Soviet Union, Anatol Bragin, has joined the project. He has extensive experience in extracellular recording from head-fixed and freely moving preparations.

During the quarter of this report, we have been working in several areas. The major results have been:

1. Fabrication of both neurochips and neuron probes has been improved. New dummy probes have been developed, but production of completed probes with electrodes was delayed by equipment problems.

2. Culture systems for hippocampal neurons at Caltech and Rutrgers have been refined, and staining methods studied.

3. Unexpected difficulty has been found in growing neurons from wells with electrodes, in neurochips. Hypotheses for why this has happened have been developed and studies have been begun to help understand the problem.

In Vitro Studies

Hippocampal cultures have been grown steadily, for two series of experiments. The first has been a study of maximizing the brightness of DiI staining. The strongest possible staining of newly dissociated and plated neurons is desirable in order to provide maximum visibility after long times of processes growing from probe neurons into host tissue.

Stock solutions of DiI were prepared in both ethanol and DMSO, at 10 mg/ml, and these were then used to add the stain to cultures. The tolerance of neurons for alcohol over a period of hours is 0.1%, which limits the DiI concentration which can be achieved in culture medium. The DMSO tolerance is about four times worse. Even though DiI is more soluble in DMSO, the best staining for a given length of time was achieved with alcohol stock solutions. The staining becomes more intense for longer times, up to 12 hours or more. However, neuron viability is compromised after about eight hours. The reason for this is that the staining must be done in serum free medium in the absence of supporting glia - which compete with the neurons and remove DiI from solution.

The best DiI staining we have obtained for hippocampal cells is not as good as was seen for SCG neurons previously, with only three hours of staining. This species dependence was not expected. Continuing studies are being made to explore two possibilities: a new dye, Sigma PKH-26, which is reputed to be as long-lasting as DiI but which stains quickly; and a technique we have been told about by Scott Fraser in which a very concentrated DiI solution in isotonic sucrose solution stains cells very brightly in very short times.

A further study with hippocampal cells has been directed toward the development of an "inverted" combination of neurons and glia. For the method of Banker, which we have been using, neurons on cover slips are placed face down a small distance away from a confluent glial bed grown on a plastic petri dish. For work with neuron probes and neurochips it is desirable to have the neurons underneath, with glial cells on cover slips placed over them. To do this, we have tried unsuccessfully to grow the glia on glass coverslips, with and without polylycine coating. Experiments with thermanox plastic cover slips are

promising, but if they do not work well enough we will have to cut circles out of plastic petri dishes.

Other *in vitro* experiments have been done with superior cervical ganglion (SCG) neurons, which we have previously used extensively for growing cultures in neurochip wells. Completed neurochips, with electrodes, have now become available, as will be discussed in the following section. A long series of failures to grow SCG cells in wells in these chips has ensued. Finally, we tried cells in dummy probes, also described below, and the growth rate was about 75% or better, as previously with dummy neurochips. The cause of the problem with the new neurochips remains unknown. It occurs with unplatinized chips as well as with platinized ones.

Experiments with cells stained with CT-orange show that neurons are indeed placed in all 16 neurochip wells, and they appear to remain there, well-stained as if alive, though not growing. We have two hypotheses: the bottom of the well is now uneven because of the electrode and silicon dioxide configuration we have been using, and cell attachment may not be taking place; or the grillwork has a large and sharp overhang which inhibits process growth out of the well. At present, to study this problem, we are preparing neurochips to obtain scanning micrographs of the junction of the grillwork and the well. In addition, we are planning to make neurochips in which the electrode configuration is simply a flat gold electrode in a flat-bottomed well. Past experience with microcircuit culture dishes in our lab has shown that cells grow well on flat electrodes, so we are optimistic that this will be a solution if the well bottom is the problem.

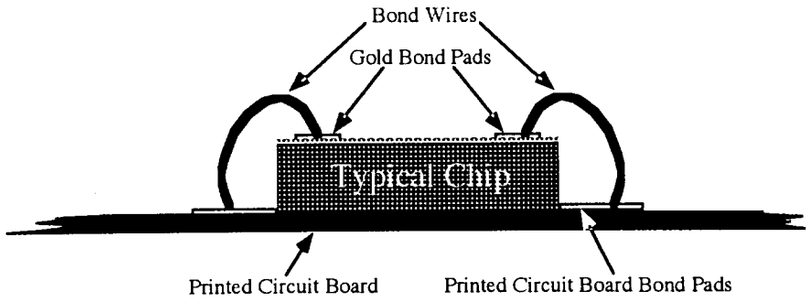
Fabrication

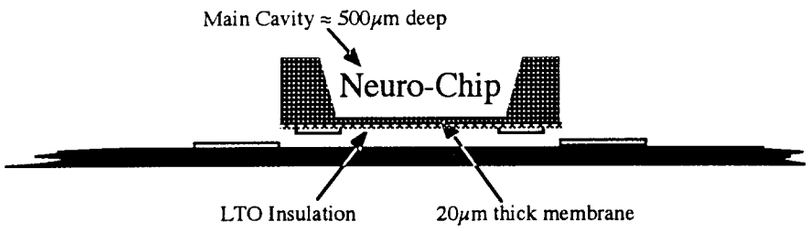
a) Neurochip platinizing

For several months we have been battling to reliably platinize the electrodes of neurochips. Until the end of this quarter, most attempts were unsuccessful and the few successes that we did enjoy were not reproducible. The reason for failure remained a mystery for some time. It was first suggested that platinization at the bottom of a well whose side walls were not insulated was impossible. It was believed that the current preferred to find its way through the silicon substrate rather than through the solution in which the chip was bathed. This theory, however, could not account for the instances in which platinization was successful.

Another explanation for the failure was that platinizing solution was not making its way to the bottom of the wells. To rule out this possibility, the chips were first filled with 95% EtOH and then rinsed with water and soaked in platinizing solution. The low surface tension of the EtOH allows it to easily fill the wells. Once wetted, the platinizing solution could then replace the EtOH via diffusion. Unfortunately, this added procedure did not result in successful platinization. Continuing along this line of thinking, it was suggested that the electrodes were still insulated from the solution by an un-etched oxide layer. After multiple attempts to remove this imagined oxide, platinization did not occur and we determined it was not the cause.

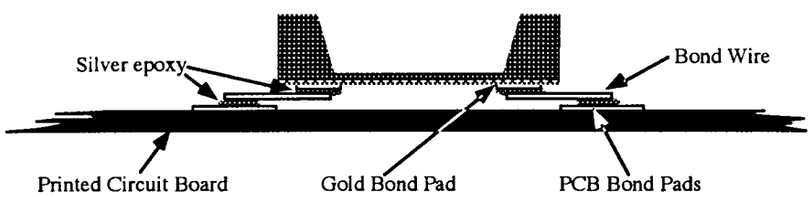
Not until we stood back and considered the entire platinizing system was the source of our troubles found. Before platinization can be attempted, a neurochip must first be mounted onto a printed circuit board (PCB) and its bond pads connected to the PCB traces. This cannot be done with the typical lead bonding used with normal chips since, to permit access to the well array, the bond pads of the neurochip must face the PCB. See diagrams below, not to scale.



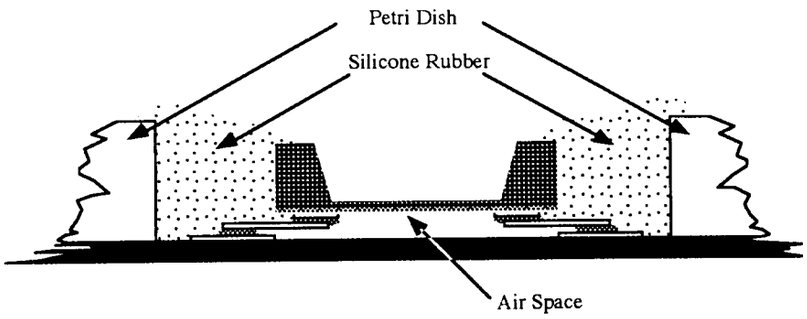


As can be seen, the orientation of the neurochip makes it impossible for a lead bonder to attach a wire to the chip and then to the PCB. Thus, the bonding must be done by hand.

The bonding method that we have adopted uses a conducting silver epoxy to first attach wire "legs" to the neurochip. These "legs" are then connected to the bond pads of the PCB. The figure below shows the ideal results of such a bonding:

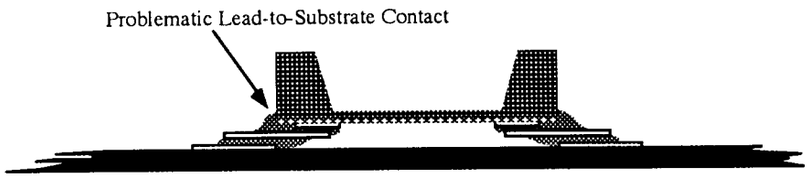


Next, a petri dish is glued onto the PCB, surrounding the neurochip, thus allowing the chip to be submerged in solutions. To protect against shorts between the leads, they are insulated by coating the area between the chip and the petri dish with silicone rubber, completing the ideal mounting procedure. A schematic of such a board is shown below.



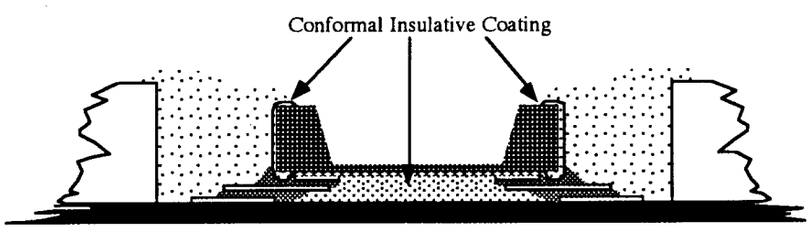
Note that mounting a chip in this manner results in an air cavity between the underside of the chip and the PCB. Theoretically, this should be fine but in actual practice, it produces two problems. First, since the thin silicon membrane of the chip is unsupported, all future work must be done very carefully so as to avoid breaking this fragile membrane. Second, while the air space should be a sealed environment, it is possible that it is not and thus liquid could find its way into it, shorting out the board. Since the air cavity is at least macroscopically sealed, such a problem cannot be confirmed without destroying the neurochip. One test that can be conducted to test this possible problem is to measure the conductivity between the 16 electrodes when the board should be dry (i.e. with no solution in the petri dish). This test has shown that the electrodes are electrically isolated from one another and so it is doubtful that solution under the chip is responsible for failed platinization.

In fact, the final answer to our problem appears to be more simple. As noted previously, silver epoxy is used to first bond wire "legs" to the neurochips and then these "legs" are then bonded to the PCB. It is this procedure that introduces the problem. Silver epoxy can run since it has a consistency similar to thin mud. While one can ensure that the epoxy does not bleed from one bond pad to another, it is much more difficult to keep it from coming in contact with the edge of the chip, which is not insulated. When this happens, current is free to flow from the wire "legs" to the silicon substrate and out through the walls of the main cavity which is also not insulated. This would be the most desirable path for the current to take since the silicon substrate has a resistance on the order of hundreds of ohms or less while the platinizing current out of a neuron well requires a drop of approximately a volt at the electrode. The situation just described is shown below:



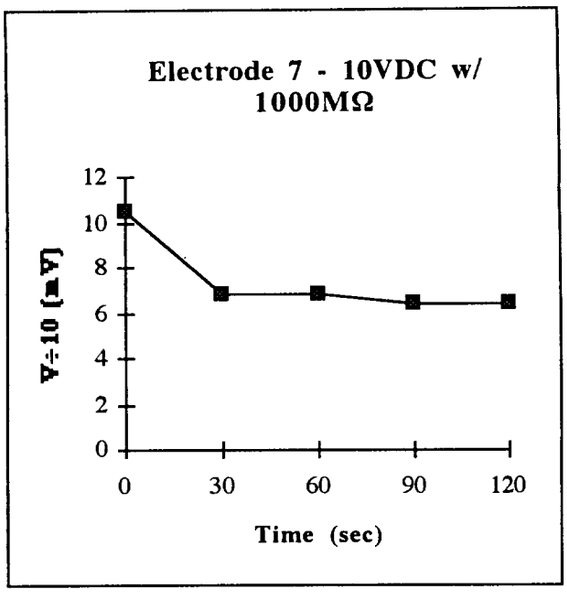
Note that this explanation can account for the fact that platinization occurs only a small percent of the time since an extremely careful mounting may result in several of the leads not being shorted to the substrate.

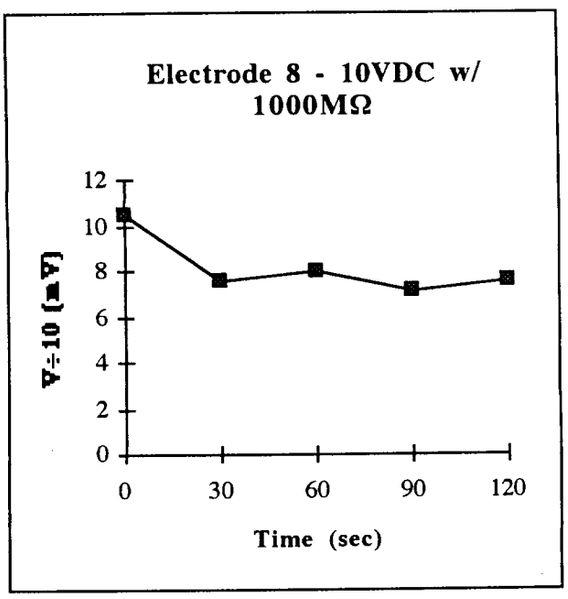
After being proposed, the problem was easily confirmed and eliminated. Using a conformal insulating material, we are able to coat the sides of the neurochips before bonding the wire "legs." This keeps the silver epoxy from coming into contact with the silicon substrate, eliminating the problem. This procedure has been used to mount several chips, all of which then went on to be successfully platinized on all electrodes. Below is a schematic of a neurochip mounted using the new procedure. Note that the air space beneath the chip no longer exists. It is now filled with conformal coating as a precaution against leakage and to strengthen the membrane.



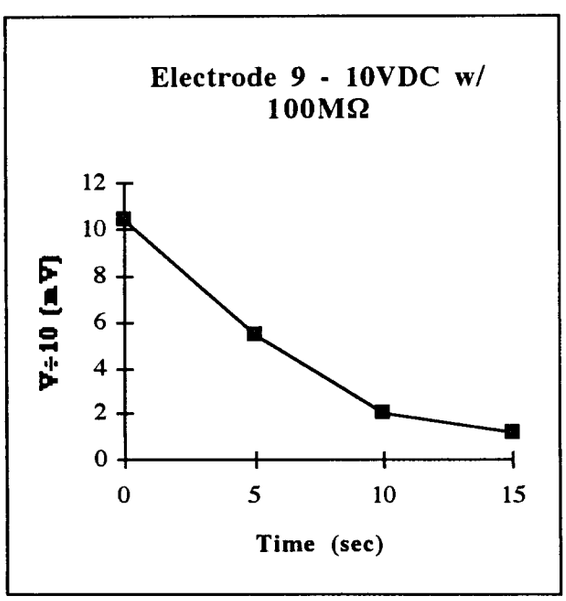
Once platinization became reproducible, the next step was to determine the parameters under which the best electrode characteristic would be produced. The two parameters easily accessible to us are platinizing current and time. To study these variables, we chose four electrodes on a chip. Through two of these, we sent 9nA and through the remaining two, we sent 80nA (these were convenient values for the setup we are using.) At specified intervals, platinization was stopped, a small 1kHz sine wave current was applied to the system and readings of the AC voltages at the electrodes were taken. The following are graphs showing the AC voltage vs. time for the four electrodes:

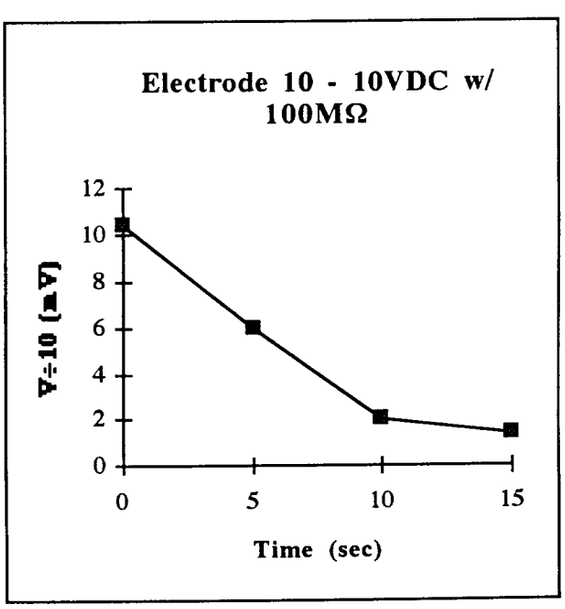
9nA Platinizing Current





80nA Platinizing Current



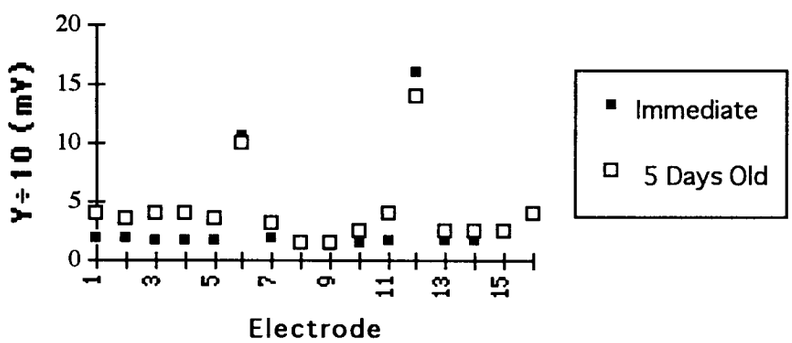


These clearly show that the 9nA current produces a minimum AC voltage of about 7mV while the 80nA platinized electrodes go as low as 1.6mV. It should be noted that the 80nA current, while producing more desirable electrical characteristics,

causes a large buildup of platinum black at the bottom of the electrode wells, a buildup that may disturb cell attachment.

A set of experiments were conducted which covered currents between 9nA and 80nA. It was found that a current of about 35nA flowing for 30 seconds produces the best results. The AC voltages of the electrodes are ≈1.8mV with no undesirable platinum black buildup. Following is a plot showing the result of a board platinized with these parameters. Overlaid is a second plot showing the AC voltages of the same board five days after it was platinized. This clearly shows that there is some degradation in the electrodes' characteristics over time but the impedances are still much lower than if they were left unplatinized. Note that electrodes 6 and 12 have high AC voltages. These values indicate that these electrodes did not platinize and are open circuits.

5 Day Old Electrode AC voltages - Board #7



The a.c. current used was 0.1 microamperes peak-to-peak, and the measured voltages across the electrodes are also peak to peak. A reading of 130 mV, the value for electrode 12, corresponds to an impedance of 1.3 megohm, which is the shunt impedance of the stray capacitance of our setup. The platinized electrode impedances are seen to be between 200K and 400K.

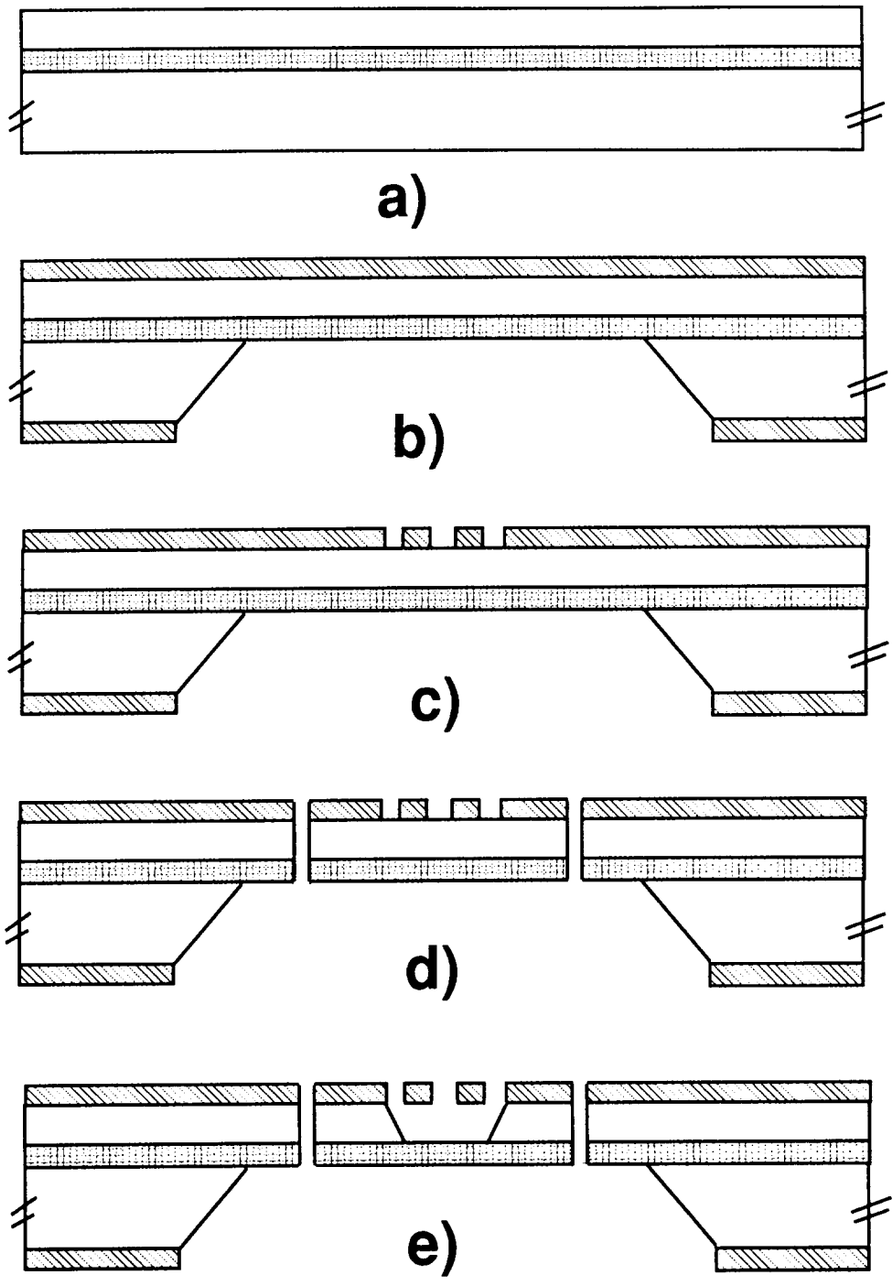
More rigorous and controlled experiments to further characterize our platinization process will be conducted in the near future, when we have a larger supply of neurochips.

b) Probe fabrication

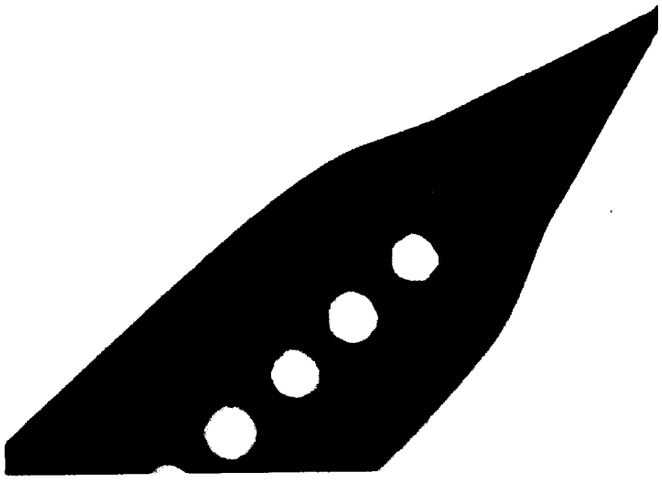
In this period a new batch of dummy probes for *in vivo* experiments was fabricated. The fabrication sequence and steps are changed with respect to the previously developed one at JPL, due to different equipment we possess. Also, we developed a process that will be very useful for the manufacturing of neuroprobes with electrodes.

The fabrication starts with epi-wafers with a 16 μm lightly-doped layer on top of a 4-μm heavily boron-doped wafer, (a) in the figure on the page following. A layer of 500 nm LPCVD low-stress Si_xN_y is formed. Photolithography (mask 1) and SF_6 plasma etching are used to pattern it at the back and open windows for subsequent EDP etching. After reaching the etch-stop layer and completing EDP etching (b), photolithography (mask2) and SF_6 RIE etching are used to define the grillwork (c). Finally, a thick layer of photoresist (~ 10 μm) is spun on the wafer and patterned to define the shape of the probe. RIE etching 20 μm deep is then used to etch the surrounding silicon, except the silicon bridge at the end of the handle (d). Additional EDP etching is used to form the neuron wells (e) and the probe is finally released by snapping it off the substrate.

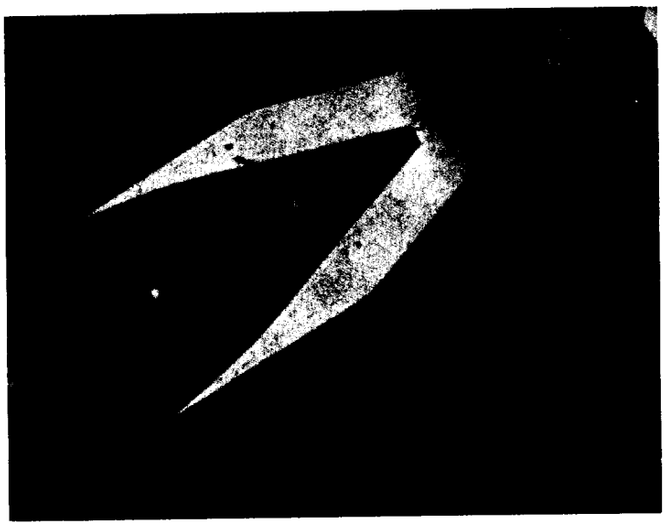
The main problem in this fabrication process was how to coat the wafer with a thick layer of photoresist so that one has a good step coverage. In the initial attempts, air bubbles were formed above the grillwork which was etched approximately 1 μm down through LPCVD silicon nitride and silicon, as shown in the figure below.


a)
b)
c)
d)
e)
-low stress LPCVD Si_xN_y
- heavily boron-doped silicon
-lightly doped silicon

These air bubbles were fatal for photoresist protection of the grillwork area during the subsequent RIE etching (instead of a dummy neuroprobe we ended up having a micromachined sewing needle), as shown in the figure below.

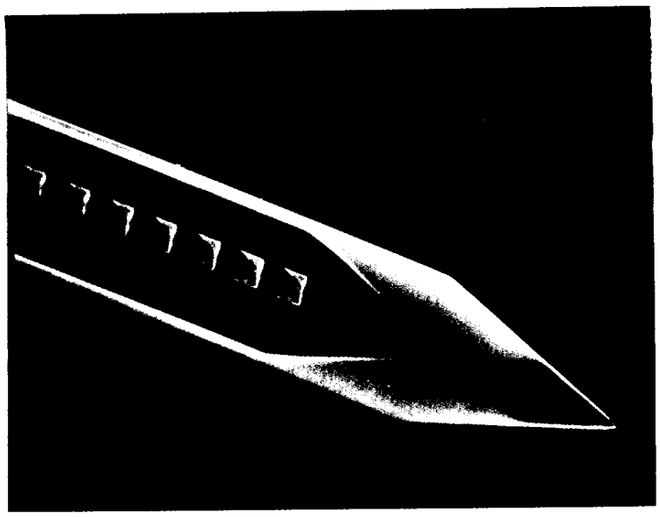


Finally, we developed a procedure for coating the wafer with photoresist without the air bubbles. An optical micrograph of the first completed dummy neuroprobe is shown below.



An effect that we didn't predict is that due to the final step of EDP etching the tip of the probe recedes. One can see that nitride membrane is overhanging the underlying silicon tip for approximately 150 microns. The consequence is that the last neuron well is too close to the tip of the probe. In order to prevent

this problem, in the next generation we moved the grillwork 150 mm further from the tip. Forty dummy neuroprobes of this type were fabricated and sent to Rutgers University. A scanning micrograph of one is shown below.



Complete neuroprobes with electrodes could not be completed because the PECVD nitride deposition system was down. (It is now working again.) However, the top side fabrication process has been improved. The alignment error of the stepper has been accurately determined and compensated, so that the alignment between gold electrode and the oxide step is now perfect, and not 1-2 mm off as it used to be. We expect that, after this correction has been introduced, the alignment between the grillwork at the top and the electrode at the bottom of the neuron well will also be much improved.

In Vivo Studies

a) Moving and survivability of cells in cultured neuron probes:

During the last quarter we devoted most of our effort to practice cell loading into dummy probes and work out tests for the in vivo labeling of the neurons. We went through a series of failures and had to identify each problem one by one.

Recently, we have established the method of glial cell coculturing in our laboratory. As discussed in our last progress report, the glia are imperative for the survival of isolated, single pyramidal cells and also improved the survival of septal neurons. First, we examined the survivability of cells plated on the surface and handle area of the probe. After 48 hrs we have seen that both septal and hippocampal cells survived well and grew out processes, although the percentage of the surviving cells was not quantified.

We loaded several dummy probes with both septal and hippocampal cells in order to estimate neuronal survival in our hands. The probes were loaded with dissociated cells and kept in the incubator for 12- to 96 hrs. As described in our earlier progress reports, we did not use the suction method for moving cells. Instead, we plated high density of cells (1,000,000 cells/ml), let them sink for 5 to 10 min, after which we moved them individually with the pusher pipette. In our hands, this proved to be a faster method for loading cells. However, we are not sure that this method is the safest one and, to date, we have no quantitative comparison with the suction method for survivability. After 5-10 min the cells can attach hard to the surface, therefore moving them with a glass pipette may be traumatic to the neurons.

In our next series of experiments we loaded cells with fluorescent DiI first before plating them, using J. Pine's protocol. The goal of these experiments is to label neurons at the time of implantation and identify them several months later in the host tissue. In the first experiments, loading cells with DiI resulted in a failure to grow the cells. In subsequent tests we left some cells on the handle area as well and found that even neurons which were not moved, therefore not

traumatized by the pipette, failed to grow. We modified the DiI loading procedure and decreased the time of centrifugation to 2 min, which procedure resulted in better survival of the neurons on the surface of the probe but not in the wells. In addition, reduction of centrifugation time produced cell clumps as well as spurious fluorescence due to the presence of debris.

Interestingly, the visibility of the DiI loaded neurons increased even with normal light illumination and we were able to clearly visualize neurons in the bottom of the wells. We have no explanation for this observation. Unfortunately, survival of the DiI loaded neurons in the wells was very poor.

It is perhaps important to emphasize that all the above experiments were done with probes fabricated previously by the Jet Propulsion Lab and several probes were reused after cleaning them. It is possible that the cleaning procedure failed to remove all debris from the bottom of the wells and that was the reason for the poor survival.

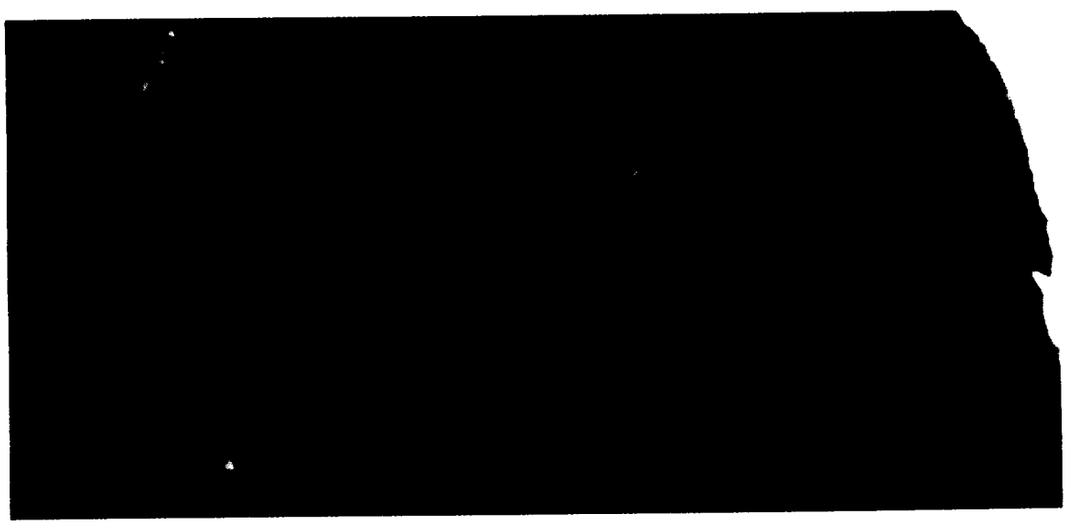
Currently, we are trying to identify the sources of our failures by the following strategy. First, recently we received new probes manufactured by the Caltech group. Since these are new, we can identify whether one source of problems was the incomplete cleaning of the probes. Second, we began experimenting with fluorescent dyes that can differentially stain live and dead cells. For this purpose we have chosen acridine orange and ethidium bromide from Molecular Probes, Inc. We plan that with such a method we can be sure that the cells that we are placing into the wells are alive. Second, and most important with such a method we should be able to monitor the possible damaging effects of physically moving neurons from the surface of the probe into the well. Although these tests were not planned, at this stage we believe they are necessary for establishing reliable moving procedures before experimenting with long-term labels.

b) Evaluation of electrode induced cell damage

Another important issue in this project is the physical-anatomical conditions of the host environment around the cultured neuron probes. During

the last three months we have investigated the histological reaction of acutely made electrode tracks in the neocortex and hippocampus. Two groups of rats were used in the experiment. In the first group of rats (short-term), a small needle was placed in the brain at four different times in four different locations. The needle (200 μm sharpened tungsten microelectrode) was inserted into the neocortex and hippocampus and removed after about a minute under surgical anesthesia. The insertions were made at two-day intervals. The rats were sacrificed 5 to 100 min after the last insertion was made. Thus, the first penetration was made six days prior to the time of perfusion, the second lesion four days and the third insertion two days before sacrifice.

The brains were stained with the Gallyas method for dark neurons that we have recently developed. Six rats were used in this group. Overall, dark neurons were observed around the tracks in both neocortex and hippocampus at all time points, although there was significant individual variability. Two rats had bleeding around the tracks and therefore could not be analyzed. Dark neurons in the hippocampus were mostly in the CA3 region and in the hilus. More often, impregnated cell bodies were not visible. However, numerous dark dendrites in the vicinity of the electrode tracks (50-100 μm) were observable, indicating that dendrites were impaired for 2 to 6 days at least around the electrode track even though this amount of trauma only rarely affected the cell bodies. The figure below shows the dark stain along the track.



We assume that these transiently impaired neurons survived, but due to the dendritic impairment reorganization of the local circuitry is expected. The

lateral track passed through the fimbria of the hippocampus. Here bundles of impregnated axons were present in all rats.

In the other group of rats (long-term; n=8) a single electrode penetration was made in each hemisphere. The electrode insertions were made the same day and the rats were perfused at different times. The rats were perfused one week (n=4), two weeks (n=2) and 1 month (n=2) after the electrode penetration. Their brains were stained for dark neurons and for microglia. Dark neurons indicate relatively acute damage (less than a week), whereas the microglia reaction has a wide time window and reveals neuronal damage for several weeks or even months.

Dark neurons were only occasionally seen and only in the 1-week group. On the other hand, microglia reaction was present at all time points around the electrode tracks in virtually every rat. The most extensive glial reaction was observed in the 1 week group. Overall, these experiments indicated that the most traumatic effect occurs with the insertion of the electrodes and the majority of the cells that die do so during the first couple of days. The dead neurons are slowly removed by microglia, which process may take several weeks.

Currently, we are extending the post-insertion period for longer than two months to determine the time required for the disappearance of microglia from around the electrode track. In addition, we have begun experiments with the dummy silicon probes that will be chronically implanted to study the long-term consequences of this material on the condition of the host brain.